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In Vitro Transcription for dgRNA V.1

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Protocol status: Working
We use this protocol and it's working

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Keywords: DASH, FLASH, cas9, dgRNA, IVT, transcription, vitro transcription for dgrna, dgrna for cas9, sgrnas for large guide rna library, large guide rna library, dgrna, sgrna, vitro transcription, crrna, rna, other crispr, cas9 protocol, components of the dual guide, dual guide, tracrrna, t7

Abstract

For FLASH, DASH, and other CRISPR-cas9 protocols, we use T7 to transcribe our crRNA and tracrRNA to make dgRNA for cas9. It is more time-, labor-, and cost-effective to make dgRNAs instead of sgRNAs for large guide RNA libraries such as those used in DASH or FLASH. The two components of the dual guides are the crRNA (containing your variable 20 nt target plus a 22 nt constant region) and the tracrRNA (a 72 nt constant region).

Guidelines

Work in an RNAse free space! If possible, work inside a PCR workstation/hood, in a pre-PCR environment.

Materials

MATERIALS

- Thermocycler
- Ethanol 100%
- NanoDrop spectrophotometer **Thermo Fisher Scientific** **Catalog #**ND-1000
- Nuclease-free water **Ambion** **Catalog #**AM9932
- Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852
- SPRI beads (homemade) or Ampure XP beads
- crRNA template (60nt) **IDT**
- tracrRNA template (90nt) **IDT**
- 10X T7 Buffer (400 mM Tris pH 7.9 - 200 mM MgCl2 - 50 mM DTT - 20 mM spermidine (Sigma 85558)) store at -80C
- T7 Enzyme (10mg/mL)
- NTP Set 100 mM Solution **Thermo Fisher Scientific** **Catalog #**R0481
- Magnetic Tube Rack for 1.5mL or 15mL tubes **Catalog #**12321D
- T7 transcription primer (18nt) **IDT**

STEP MATERIALS

- crRNA template (60nt) **IDT**
- T7 transcription primer (18nt) **IDT**
- tracrRNA template (90nt) **IDT**
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- Nuclease-free water **Ambion** **Catalog #**AM9932
- crRNA template (60nt) **IDT**
- Nuclease-free water **Ambion** **Catalog #**AM9932
- SPRI beads (homemade) or Ampure XP beads
- Ethanol 100%

Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852

Agilent Small RNA Bioanalyzer kit **Catalog #**5067-1548

- *NTP quality varies from one vendor to another. We have had consistent success with Thermo cat # r0481 and Life Tech AM81110G, -20G, -30G, and -40G, used at a final concentration of 1 mM each*
- *We purify our own T7, and experiments should be optimized for each batch of T7, or for a commercial T7.*

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- Nuclease-free water **Ambion** **Catalog #**AM9932
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- tracrRNA template (90nt) **IDT**
- Thermocycler
- crRNA template (60nt) **IDT**
- 10X T7 Buffer (400 mM Tris pH 7.9 - 200 mM MgCl2 - 50 mM DTT - 20 mM spermidine (Sigma 85558)) store at -80C
- T7 Enzyme (10mg/mL)
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- T7 transcription primer (18nt) **IDT**

T7 transcription primer (18nt) **IDT**

crRNA template (60nt) **IDT**

T7 transcription primer (18nt) **IDT**

tracrRNA template (90nt) **IDT**

Nuclease-free water **Ambion** **Catalog #**AM9932

crRNA template (60nt) **IDT**

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SPRI beads (homemade) or Ampure XP beads

Ethanol 100%

Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852

Agilent Small RNA Bioanalyzer kit **Catalog #**5067-1548

Troubleshooting

Before start

Designing the crRNA(s): (contains your target sequence)

□ *S. pyogenes* cas9 requires a 20-nt target directly 5′ to a PAM motif "NGG" (where N is any nucleotide). The NGG is not present in the guide RNA itself. So when choosing a target you are looking for a sequence that matches the following pattern (and don't forget that you can target either strand):

5′----**NNNNNNNNNNNNNNNNNNNN**NGG----3′

or 5′----CCN**NNNNNNNNNNNNNNNNNNNN**----3′

where the 20 Ns in bold are your target site. Cas9 will cut between the 17thand 18th nt of the target, yielding the following products:

5′----**NNNNNNNNNNNNNNNNNN**3′ 5′**NNN**NGG----3′

or 5′----CCN**NNN**3′ 5′**NNNNNNNNNNNNNNNNNN**----5′

□ The sequence of each crRNA should be as follows, with the Ns replaced by your 20 nt target:

TAATACGACTCACTATAGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTATGCTGTTTTG

The underlined portion is the T7 transcription site. T7 only requires its own 18 nt binding site to be double-stranded; the rest of the template can be single stranded. Thus the template can be constructed by purchasing two oligos from IDT: the reverse complement of the 60 nt sequence listed above, plus an 18 nt oligo to make the T7 site double stranded:

60mer reverse complement:
CAAAACAGCATAGCTCTAAAACNNNNNNNNNNNNNNNNNNNNCTATAGTGAGTCGTATTA

18mer T7:
TAATACGACTCACTATAG

The tracrRNA: (constant for all dgRNA)

□ The sequence of the tracrRNA template should be as follows:

TAATACGACTCACTATAGGACAGCATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTT

Just as with the crRNA, only the T7 binding site needs to be double stranded, so the following two oligos can be purchased from IDT:

90mer reverse complement:
AAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATGCTGTCCTATAGTGAGTCGTATTA

18mer T7:
TAATACGACTCACTATAG

Annealing T7 to crRNA and tracrRNA template

1 Pool your crRNA in equimolar amounts. Usually, we order 96-well plates of 96 crRNA templates from IDT, with the oligos diluted in water at a concentration of 10µM

crRNA template (60nt) **IDT**

2 Add an equimolar amount of T7 to your crRNA pool. For example, reconstitute T7 to 10µM, and pool 500µL of your 10µM crRNA pool with 500µL of your T7 at 10µM.

T7 transcription primer (18nt) **IDT**

3 Add an equimolar amount of T7 to your tracr RNA. For example, if you have reconstituted your tracrRNA to 100µM, pool 500µL of your tracrRNA at 100µM to 500µL of your T7 at 100µM.

tracrRNA template (90nt) **IDT**

T7 transcription primer (18nt) **IDT**

4 Anneal tracrRNA + T7 and crRNA + T7 by heating to 95 °C on a heat block or thermocycler for 00:02:00 and allowing them to cool to room temperature slowly on the bench

Prepare for IVT reaction

5 Nanodrop or Qubit your tracrRNA and crRNA using ssDNA setting or kit.

NanoDrop spectrophotometer **Thermo Fisher Scientific Catalog #**ND-1000

6 Dilute your tracrRNA with T7 annealed to 800ng/µL with water. Dilute your crRNA with T7 to 40ng/µL with water.

Note

Can store these as aliquots at -20 °C

In Vitro Transcription

7 Make all reagents are at room temperature. Prepare the reaction mixtures below in the order specified. DO NOT prepare the reaction on ice, as some components are prone to precipitation.

8 Mix NTPs together in equimolar amounts to have enough for the following reactions. For example, mix 200µL A NTP at 100mM, 200µL C NTP at 100mM, 200µL G NTP at 100mM, and 200µL U NTP at 100mM for a final solution of 25mM each.

NTP Set 100 mM Solution **Thermo Fisher Scientific Catalog #**R0481

9 Prepare a small amount of 1X T7 buffer (200µL for the reaction below). Dilute your T7 enzyme to 100µg/mL in 1X T7 buffer.

10X T7 Buffer (400 mM Tris pH 7.9 - 200 mM MgCl2 - 50 mM DTT - 20 mM spermidine (Sigma 85558)) store at -80C

T7 Enzyme (10mg/mL)

10 Prepare crRNA mixture by adding the following reagents to a 1.5mL tube in order.

crRNA template (60nt) **IDT**

Nuclease-free water **Ambion Catalog #**AM9932

	Volume	crRNA
	1X	
	380µL	RNAse-free water
	120µL	10X T7 buffer*
	300µL	NTPs 25mM each
	100µL	T7 enzyme (1:100 diluted in 1X T7 buffer, final conc: 100µg/mL)
	100µL / 4µg	crRNA template with T7 annealed, 40ng/µL
	1mL	**TOTAL**

*Experiments indicated that treating the 10x T7 buffer like 8.3x improved yields

11 Prepare tracrRNA mixture by adding the following reagents to a 1.5mL tube in order.

tracrRNA template (90nt) **IDT**

Nuclease-free water **Ambion Catalog #**AM9932

	Volume	tracrRNA
	1X	
	470µL	RNAse-free water
	120µL	10X T7 buffer*
	300µL	NTPs 25mM each
	100µL	T7 enzyme (1:100 diluted in 1X T7

		buffer, final conc: 100µg/mL)
	10µL / 8 µg	tracrRNA template with T7 annealed, 800ng/µL
	1mL	TOTAL

*Experiments indicated that treating the 10x T7 buffer like 8.3x improved yields

12 Incubate at 37 °C for 02:00:00 and proceed immediately to purification.

RNA Purification with SPRI beads

13 Use homemade SPRI beads or Ampure beads to purify gRNAs after transcription.

SPRI beads (homemade) or Ampure XP beads

Note

Note: Alternatively, RNA may be purified using a Zymo RNA Clean & Concentrator-5 or -25 Kit (Zymo Research R1015)

14 Equilibrate SPRI beads to room temperature.

15 For every 200 µL of IVT reaction, add 300µL of 100% ethanol. The solution should turn a cloudy white (precipitation of RNA) upon addition of ethanol. This step helps the short RNAs bind to the SPRI beads. This can be done in a 15mL tube or several 1.5mL tubes.

Ethanol 100%

16 Add 500µL of SPRI beads to the solution of IVT reaction and ethanol and mix well by inverting or pipetting with a P1000.

17 Incubate at room temperature for 00:05:00 .

18 Divide this mixture up into an appropriate number of 1.5mL Lo-Bind Eppendorf tubes and place on a 1.5 mL magnetic separation rack OR use a 15mL magnetic tube rack.

19 Wait 00:05:00 to allow the beads to separate if using a 1.5mL rack OR 00:15:00 if using a 15mL rack.

20 Remove and discard the supernatant.

21 Rinse the beads with 1mL of 80% ethanol if using a 1.5mL tube or ~10mL of 80% ethanol if using the 15mL tube. It is not necessary to resuspend the beads.

22 Wait 00:01:00 then remove and discard the ethanol.

23 Repeat the wash step as described above. (Add the same amount of 80% ethanol, wait 00:01:00 , then discard the ethanol.)

24 Remove residual ethanol that collects at the bottom of the tube by using a P200 or P20.

25 Air dry the beads for 00:05:00 in a 1.5mL tube or 00:15:00 in a 15mL tube, or until the beads lose their glossy appearance. Sufficiently dry beads will appear matte. Be careful not to let the beads get too dry (appearing cracked or dusty).

26 Elute the RNA by resuspending the beads with an appropriate amount of nuclease-free H2O depending on the desired volume and concentration. For DASH guides which need to be at a final concentration of 40µM, lower is better (80µL per 1X reaction). For FLASH guides which need to be at a final concentration of 4µM, a higher volume can be used.

27 Allow the RNA to elute off the beads by incubating at room temperature for 00:05:00 .

28 If necessary, pulse-spin the tubes to collect any liquid along the sides of the tubes.

29 Place the tubes on the magnetic rack and allow them to separate until water is clear. This will take 2-5 minutes for a 1.5mL tube, 5-10 minutes for a 15mL tube.

30 Collect the eluted RNA, being careful not to take up beads. (Eg. If eluted in 80uL, collect 75µL).

Quantify, anneal and aliquot dgRNA

31 Using the HS RNA Qubit kit, quantify 1µL of the eluted tracrRNA and 1µL of the eluted crRNA. Follow standard HS RNA Qubit protocol.

Qubit RNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32852

32 crRNA: For DASH, dilute the stock crRNA to 1100 ng/µL. This is equivalent to 80 µM. For FLASH or other lower concentration needs, dilute to 110 ng/µL or 8µM

33 tracrRNA: For DASH, dilute the stock crRNA to 1900 ng/µL. This is equivalent to 80 µM. For FLASH or other lower concentration needs, dilute to 190 ng/µL or 8µM

34 If you want to check purity, run your 1:100 dilutions on a small RNA chip on the bioanalyzer immediately after denaturing them by heating to 95 °C for 00:03:00 . The crRNA and tracrRNA are 42 nt and 72 nt long, respectively.

Agilent Small RNA Bioanalyzer kit **Catalog #**5067-1548

35 To form the dgRNA complex, mix together equilmolar amounts of crRNA and tracrRNA (equal volumes of the 80 µM crRNA stock and the 80 µM tracrRNA stock), heat to 95 °C for 00:00:30 and then cool slowly on the bench.

36 Store dgRNA at -80 °C in small aliquots in order to avoid freeze-thaws. If there is crRNA or tracrRNA left over, freeze them separately -- the dgRNA complex can be

formed in the same way (95 °C for 00:00:30) immediately prior to complexing with Cas9.